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Development of a quantitative real-time PCR for the detection of canine respiratory coronavirus

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A B S T R A C T

Canine respiratory coronavirus (CRCoV) has been detected recently in dogs with canine infectious respiratory disease and is involved in the clinical disease complex. CRCoV is a group 2 coronavirus most closely related to bovine coronavirus and human coronavirus OC43. A real-time PCR assay was developed for the detection of CRCoV. The assay was validated against cell culture grown virus and shown to have a high level of sensitivity. A range of tissue samples were collected from dogs at a re-homing centre with a history of endemic respiratory disease. The samples were tested using a conventional nested PCR assay and CRCoV was quantitated by real-time PCR. CRCoV was detected most frequently in the nasal mucosa, nasal tonsil and trachea. It was also detected in the lung, and bronchial lymph node. Of the enteric tissues, only one mesenteric lymph node sample was positive. In addition two colon samples were positive for CRCoV by nested PCR only. In conclusion, CRCoV appears to infect the upper respiratory tract preferentially. The CRCoV real-time PCR assay has proved to be a highly specific and sensitive assay that can be applied for diagnostic purposes as well as to investigate further the tissue tropism of CRCoV.

1. Introduction

Canine infectious respiratory disease (CIRD) is a disease complex which occurs frequently in densely housed dog populations such as in re-homing, training and boarding kennels. The disease is characterised by a dry hacking cough with a recovery period of 1–3 weeks, but can progress to a severe bronchopneumonia which may be fatal (Appel and Binn, 1987). Many microbiological agents have been associated with CIRD; including *Bordetella bronchiseptica*, canine parainfluenza virus; canine adenovirus-2; canine herpesvirus and *Mycoplasma* spp. which may act alone or in synergy to cause disease (Binn et al., 1967; Ditchfield et al., 1962; Karpas et al., 1968; Keil and Fenwick, 1998; Randolph et al., 1993). However, despite widespread vaccination against a number of these agents, CIRD has remained a persistent problem (Erles et al., 2004).

Recently, a novel coronavirus; canine respiratory coronavirus (CRCoV); was detected in respiratory samples collected from dogs in a UK re-homing centre with a high incidence of CIRD (Erles et al., 2003), and the role of CRCoV in CIRD is currently under investigation.

Coronaviruses are large enveloped viruses containing a single stranded positive-sense RNA genome of approximately 30 kb which encodes a polymerase complex and several structural (spike (S), envelope (E), membrane (M), nucleocapsid (N), and in group two coronaviruses only a haemagglutinin esterase (HE)) and non-structural proteins. Currently coronaviruses are divided into three groups based on genetic and antigenic traits (Gonzalez et al., 2003).

Phylogenetic analysis places CRCoV in group 2 of the *Coronaviridae* family related most closely to bovine coronavirus (BCoV) and human coronavirus OC43 (OC43) (Erles et al., 2003). Serological studies have shown CRCoV to be present in the UK, Ireland, Italy, USA and Japan (Decaro et al., 2007; Erles and Brownlie, 2005; Kaneshima et al., 2006; Priestnall et al., 2006, 2007).

Current detection methods for CRCoV include virus isolation and detection of viral RNA by reverse transcription polymerase chain reaction (RT-PCR) (Erles et al., 2007, 2003). Cell culture isolation of CRCoV is difficult and time consuming, and therefore lacks the sensitivity and speed required for high-throughput screening (Erles et al., 2003; Kaneshima et al., 2006). Molecular assays such as RT-PCR are faster and offer increased sensitivity and specificity over other detection methods (Tang, 2003; Wang et al., 1999), therefore providing the best opportunity for the detection of CRCoV. The current RT-PCR method for the detection of CRCoV (Erles et al., 2003), however does not enable quantitation of viral load.

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Studies which focus on the assessment of tissue tropism, viral load and shedding are vital for understanding the pathogenesis of CRCoV. In a clinical setting this has particular relevance for monitoring the course of disease, disease management and clinical outcome, therefore the development of a quantitative PCR (qPCR) is essential.

Real-time qPCR has many advantages over conventional nested PCR methods. In addition to increased speed and often sensitivity, real-time qPCR offers the ability to quantitate viral load in a closed tube system which requires no further manipulation, therefore also reducing the potential contamination problems encountered commonly with nested PCR assays (Bustin, 2000; Bustin and Mueller, 2005). One qPCR method available currently, which may be used for the quantitation of CRCoV, uses highly degenerate primers which enables the detection of coronaviruses from all three coronavirus groups (Escutenaire et al., 2007). As such this assay is unable to distinguish between CRCoV and the other coronavirus which infects dogs, canine coronavirus (CCoV); a group one virus, which causes enteric disease (Binn et al., 1974).

The present study describes the development of a real-time qPCR for CRCoV and the quantitation of CRCoV RNA loads in various post-mortem tissues collected from dogs during naturally occurring infections with the virus. These data are important for defining the natural history and tropism of CRCoV infection, and improving understanding of the basic pathology of CRCoV.

2. Materials and methods

2.1. Cells and viruses

A 75-cm^2 culture flask containing HRT-18 cells (ECACC), was washed with 2 ml of serum-free RPMI containing 1 μg/ml trypsin (Sigma, Gillingham, UK) and inoculated with 1×10^6 $TCID_{50}$ of CRCoV isolate 4182 (Erles et al., 2007). After 1 h at 37 °C the inoculum was removed and replaced with 10 ml of serum-free RPMI containing 1 μg/ml trypsin. After 5 days the virus laden cell culture supernatant was titrated as described previously (Erles et al., 2007).

2.2. Study population and sample collection

The carcases of 10 dogs from a re-homing kennel were used in this investigation. One dog was suffering from cough and nasal discharge at the time of euthanasia. The other nine dogs did not exhibit obvious signs of respiratory disease or any other clinical conditions. Necropsies were performed within 4 h of death. Samples from the following tissues were harvested for storage at −70 °C: nasal mucosa, nasal tonsil, palatine tonsil, mid-trachea, right apical lung lobe, left diaphragmatic lung lobe, bronchial lymph node, liver, spleen, colon and anterior mesenteric lymph node (within the root of the mesentery). Each tissue sample was taken using a new set of sterile instruments to avoid cross-contamination. Tracheal samples collected from an additional 38 dogs from the same re-homing centre, all with evidence of respiratory disease, were also collected in the same manner. All the dogs used in this study were euthanized solely on grounds of being unsuitable for re-homing due to aggressive behaviour.

2.3. RNA extraction from cell culture derived virus

RNA was extracted using the RNeasy Mini Kit (Qiagen, Crawley, UK) from 200 μl of CRCoV infected cell culture supernatant samples as recommended by the manufacturer.

2.4. RNA extraction from clinical samples

RNA was extracted using TRiReagent (Sigma) from approximately 25–50 mg of homogenised canine tissue samples as recommended by the manufacturer. RNA was treated with RQ1 RNAse free DNAse (Promega, Southampton, UK) to remove genomic DNA according to the manufacturer's protocol.

2.5. cDNA synthesis and quantification

RNA was transcribed into cDNA using Random Hexameres (GE Healthcare, Little Chalfont, UK) and Improm II reverse transcriptase (Promega) according to the manufacturer's protocol. Total cDNA was quantified using the Quant-iT^{TM} PicoGreen® Assay Kit (Invitrogen, Paisley, UK) which selectively detects dsDNA (removed from these samples by DNase treatment (see Section 2.4)) and DNA–RNA hybrids (cDNA) (Seville et al., 1996). The assay was performed using a modification of a previously published protocol (Whelan et al., 2003). Briefly the λ DNA standard was diluted in 1× Tris–EDTA (TE) in a fivefold dilution series from 1 μg/ml to 1.6 ng/ml. cDNA samples were diluted 1:50 in 1× TE. 50 μl per well of each standard/cDNA sample was aliquoted in duplicate in a 96 well plate. The PicoGreen dsDNA quantitation reagent (Invitrogen) was diluted 1:200 in 1× TE and 50 μl was added to each well, therefore making a further 1:2 dilution of the standards and samples. The fluorescence was measured using the SPECTRAmax M2 Plate reader (Molecular devices) with an excitation at 480 nm and emission at 520 nm. The linear standard curve was constructed for the λ DNA standard concentration vs. fluorescence units. The concentration of the cDNA was calculated from the linear equation after adjusting for the dilution.

2.6. Conventional PCR for GapDH

Prior to analysis for CRCoV all samples were tested for the presence of the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GapDH) by conventional PCR as described previously (Grone et al., 1996). The PCR products were resolved on 1.5% agarose gels containing 0.5 μg/ml of ethidium bromide and visualised under UV illumination using Gel Doc 2000 (Bio-Rad, Hemel Hempstead, UK) for image capture.

2.7. Conventional CRCoV spike gene PCR

Clinical samples were analysed for CRCoV using the conventional nested spike gene PCR as described previously (Erles et al., 2003). The PCR products were resolved on 1.5% agarose gels containing 0.5 μg/ml of ethidium bromide and visualised under UV illumination using Gel Doc 2000 (Bio-Rad) for image capture.

2.8. Real-time qPCR primer design

Due to the limited availability of CRCoV sequence data the CRCoV nucleocapsid gene was selected as the target for the real-time PCR as it is highly conserved within each coronavirus group, thus ensuring the detection of variant CRCoV strains, whilst being sufficiently divergent to differentiate between the three coronavirus groups, and therefore CRCoV and CCoV (Gonzalez et al., 2003). The primers CRCoV NF3 (forward 5′-CCCTACTATTCTTGGTT-3′) and CRCoV NR4 (reverse 5′-CGTCTGTTGTGTCTGTACC-3′) were designed against the only available nucleotide sequence for the CRCoV nucleocapsid gene (GenBank accession no: DQ682406) which was aligned with sequences from other group 2 coronaviruses previously published in the GenBank database (GenBank accession no: U00735, AF220295, AY585228, AY585229, NC007732, DQ011855), a group 1 CCoV sequence (GenBank accession no: AY548235) and